The EMBO Journal vol.15 no.24 pp.6951–6959, 1996

Mutational analysis of VAMP domains implicated in Ca^{2+}-induced insulin exocytosis

Romano Regazzi[1], Karin Sadoul[2], Paolo Meda[3], Regis B.Kelly[4], Philippe A.Halban[2] and Claes B.Wollheim

Division de Biochimie Clinique, Département de Medicine Interne, [2]Laboratoires Louis Jeantet and [3]Département de Morphologie, Université de Genève, CH-1211 Genève 4, Switzerland and [4]Department of Biochemistry and Biophysics, Hormone Research Institute, University of California, San Francisco, CA 94143-0534, USA

[1]Corresponding author

Vesicle-associated membrane protein-2 (VAMP-2) and cellubrevin are associated with the membrane of insulin-containing secretory granules and of γ-aminobutyric acid (GABA)-containing synaptic-like vesicles of pancreatic β-cells. We found that a point mutation in VAMP-2 preventing targeting to synaptic vesicles also impairs the localization on insulin-containing secretory granules, suggesting a similar requirement for vesicular targeting. Tetanus toxin (TeTx) treatment of permeabilized HIT-T15 cells leads to the proteolytic cleavage of VAMP-2 and cellubrevin and causes the inhibition of Ca^{2+}-triggered insulin exocytosis. Transient transfection of HIT-T15 cells with VAMP-1, VAMP-2 or cellubrevin made resistant to the proteolytic action of TeTx by amino acid replacements in the cleavage site restored Ca^{2+}-stimulated secretion. Wild-type VAMP-2, wild-type cellubrevin or a mutant of VAMP-2 resistant to TeTx but not targeted to secretory granules were unable to rescue Ca^{2+}-evoked insulin release. The transmembrane domain and the N-terminal region of VAMP-2 were not essential for the recovery of stimulated exocytosis, but deletions preventing the binding to SNAP-25 and/or to syntaxin I rendered the protein inactive in the reconstitution assay. Mutations of putative phosphorylation sites or of negatively charged amino acids in the SNARE motif recognized by clostridial toxins had no effect on the ability of VAMP-2 to mediate Ca^{2+}-triggered secretion. We conclude that: (i) both VAMP-2 and cellubrevin can participate in the exocytosis of insulin; (ii) the interaction of VAMP-2 with syntaxin and SNAP-25 is required for docking and/or fusion of secretory granules with the plasma membrane; and (iii) the phosphorylation of VAMP-2 is not essential for Ca^{2+}-stimulated insulin exocytosis.

Keywords: islets of Langerhans/neurotoxins/pancreas/secretion/targeting

Introduction

Insulin secretion from pancreatic β-cells plays an essential role in the achievement of blood glucose homeostasis. The molecular components of the machinery targeting insulin-containing secretory granules to the plasma membrane and regulating their fusion during the exocytotic process are still largely unknown. However, the general mechanisms of vesicle docking and fusion are conserved throughout evolution (Ferro-Novick and Jahn, 1994; Rothman, 1994). According to the SNARE hypothesis, the secretory vesicles are targeted to their acceptor compartment as a result of the specific interaction between a class of proteins located on the vesicle membrane and called v-SNAREs (vesicular SNAP receptors) and their specific partners on the target membrane, the t-SNAREs (target SNAP receptors) (Söllner *et al.*, 1993a; Rothman and Warren, 1994). The formation of the complex between v- and t-SNAREs serves as an assembly site for other cytosolic components of the intracellular fusion machinery (Söllner *et al.*, 1993a,b). The VAMPs (Vesicle-Associated Membrane Proteins), also known as synaptobrevins, belong to the v-SNARE class. The three VAMP isoforms known (Elferink *et al.*, 1989; McMahon *et al.*, 1993), VAMP-1, VAMP-2 and cellubrevin, have a conserved region in their cytoplasmic domain adjacent to the C-terminal transmembrane anchor predicted to form two amphipathic α-helices (Dascher *et al.*, 1991). Helix 1 (amino acids 39–53 in VAMP-2) contains a signal essential for the targeting of the protein to synaptic vesicles (Grote *et al.*, 1995). Helix 2 (amino acids 60–88) has a high probability of forming coiled-coil structures (Lupas *et al.*, 1991). Several lines of evidence implicate VAMPs in the process of vesicle docking and fusion. Thus, some of the clostridial neurotoxins that block neurotransmitter release selectively cleave VAMPs (Schiavo *et al.*, 1992; Niemann *et al.*, 1994; Rossetto *et al.*, 1995), and *Saccharomyces cerevisiae* lacking the yeast homologs of VAMPs are secretion deficient and accumulate post-Golgi vesicles (Protopopov *et al.*, 1993).

The respective roles of VAMP isoforms are still unclear. Cellubrevin is expressed ubiquitously and is localized on endosome-derived vesicles which undergo constitutive exocytosis (McMahon *et al.*, 1993; Galli *et al.*, 1994). This raises the possibility that cellubrevin could function in a distinct secretory pathway.

Insulin-secreting cells express several proteins of the v- and t-SNARE classes (Jacobsson *et al.*, 1994; Regazzi *et al.*, 1995; Sadoul *et al.*, 1995; Wheeler *et al.*, 1996; Wollheim *et al.*, 1996). With regard to the v-SNAREs, insulin-secreting cells express VAMP-2 and cellubrevin, but not VAMP-1 (Jacobsson *et al.*, 1994; Regazzi *et al.*, 1995; Rossetto *et al.*, 1996; Wheeler *et al.*, 1996). Both VAMP-2 and cellubrevin are associated with insulin-containing secretory granules as well as with synaptic-like microvesicles containing the neurotransmitter γ-aminobutyric acid (GABA; Regazzi *et al.*, 1995). VAMP-2 and/or cellubrevin appear to be essential for

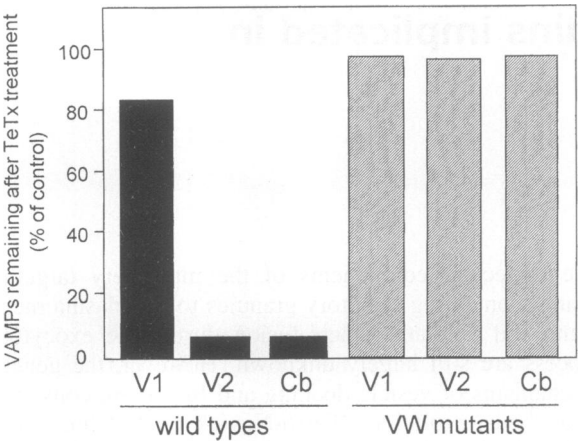

Fig. 1. Generation of VAMP mutants insensitive to TeTx. Wild-type rat VAMP-1 (V1), VAMP-2 (V2) and cellubrevin (Cb) and the variants of these proteins (VW) mutated at position 79 (Phe→Trp), 76/77 (Gln/Phe→Val/Trp) and 63/64 (Gln/Phe→Val/Trp), respectively, were produced by *in vitro* translation in the presence of [^{35}S]methionine. The radiolabeled proteins were incubated for 18 h in the presence or absence of 100 nM light chain TeTx. After the incubation with the toxin, the proteins were analyzed by SDS–PAGE, and the percentage of each of the VAMPs remaining was evaluated by densitometric scanning of the autoradiography films.

Ca^{2+}-induced insulin exocytosis. Thus, pre-treatment of permeabilized insulin-secreting cells with tetanus toxin (TeTx) or with botulinum neurotoxin type B (BoNT B), that selectively cleave VAMP-2 and cellubrevin, produces a concomitant inhibition of secretion (Regazzi *et al.*, 1995; Wheeler *et al.*, 1996).

In this study, the implication of the individual VAMP isoforms in the control of insulin secretion was investigated through restoration of Ca^{2+}-induced exocytosis in TeTx-treated cells. This was achieved by transfecting insulin-secreting cells with mutants of VAMPs that are resistant to TeTx cleavage. Using this approach, the three VAMP isoforms were found to have similar functions and to participate in the control of insulin exocytosis. In addition, we defined the regions of VAMP-2 that are essential for its targeting to secretory granules and for the regulation of insulin exocytosis.

Results

Generation of VAMP mutants insensitive to TeTx

To determine the role of VAMPs in the control of insulin secretion, we generated mutants insensitive to proteolytic cleavage by TeTx and tested whether these proteins would restore Ca^{2+}-induced exocytosis. *In vitro* synthesized wild-type rat VAMP-2 and cellubrevin incubated in the presence of TeTx light chain (100 nM) for 18 h were cleaved efficiently, but rat VAMP-1 that contains a Val instead of a Gln in the cleavage site was only poorly proteolysed (Figure 1). Rat VAMP-1 became completely resistant to TeTx cleavage when the second amino acid at the cleavage site, Phe79, was replaced by Trp (Figure 1). To produce mutants of VAMP-2 and cellubrevin resistant to TeTx treatment, Gln76 and Phe77 in VAMP-2 and Gln63 and Phe64 in cellubrevin were substituted by Val and Trp, respectively. As expected, these mutants (VW) were not cleaved by the toxin (Figure 1).

Reconstitution of Ca^{2+}-stimulated exocytosis in TeTx-treated cells

Next, we analyzed which of the VAMP isoforms was able to rescue Ca^{2+}-induced exocytosis in TeTx-treated cells. Experiments performed with *in vitro* translated proteins demonstrated that the VW mutants do not inhibit the cleavage of wild-type VAMPs competitively. Thus, the presence of an excess of cellubrevin VW mutant does not decrease the cleavage of wild-type VAMP-2 significantly (not shown). To reconstitute stimulated insulin secretion in TeTx-treated cells, the hamster β-cell line HIT-T15 was transiently co-transfected with a plasmid encoding human proinsulin as a marker for hormone secretion and with the different constructs to be studied. We have demonstrated previously that, under our experimental conditions, the large majority (>90%) of the transfected cells co-express the proteins encoded by two such plasmids (Lang *et al.*, 1995; Regazzi *et al.*, 1996). Thus, by specifically measuring human C-peptide release from the hamster cells, we can determine exocytosis from the subpopulation of transfected cells (10–15%) without the need for separating them from non-transfected cells (Lang *et al.*, 1995; Regazzi *et al.*, 1996). In a first set of experiments, we found that transfection with 12.5 μg of VAMP cDNA had no effect on basal C-peptide release but completely prevented Ca^{2+}-stimulated exocytosis in permeabilized cells (not shown). We therefore decreased the amount of DNA used to transfect the cells to 1.5 μg. Under these conditions, Ca^{2+}-triggered exocytosis in permeabilized HIT-T15 cells was comparable with that of untransfected cells. To evaluate the level of overexpression achieved under these conditions, HIT-T15 cells were transiently co-transfected with human CD4 and with a plasmid containing an epitope-tagged version of VAMP-2 (VAMP-2TAg) (Grote *et al.*, 1995). Two days after transfection, the cells co-expressing CD4 and VAMP-2TAg were purified on magnetic beads coated with an antibody against CD4. Western blotting using extracts of the purified cells indicated that the transfected cells express 3- to 4-fold more VAMP-2TAg than endogenous VAMP-2 (not shown).

HIT-T15 cells transiently transfected with human proinsulin and with vector alone or containing the cDNAs for the different VAMPs were permeabilized with streptolysin-O (SL-O) and pre-incubated in the presence or absence of 50 nM TeTx. Ca^{2+}-induced exocytosis in cells transfected with the vector alone and pre-treated with TeTx was ~25% of the secretion obtained in cells not exposed to the neurotoxin (Figure 2). Similar inhibition was observed in untransfected cells (not shown) and in cells expressing wild-type VAMP-2 and cellubrevin. In cells producing wild-type VAMP-1, which is only poorly proteolysed by TeTx (see Figure 1), Ca^{2+}-induced secretion was restored to >80% of the level obtained in the absence of the neurotoxin (Figure 2). In cells transfected with the TeTx-insensitive VW mutants, each of the three VAMP isoforms was able to reconstitute Ca^{2+}-evoked exocytosis (Figure 2).

In cells pre-treated with 100 nM TeTx, Ca^{2+}-triggered C-peptide secretion decreased to ~15% of the control (Figure 3). Under these conditions, the VW mutants of cellubrevin and of VAMP-2 reconstituted exocytosis to ~60% of the level obtained in cells not exposed to the neurotoxin (Figure 3). The same recovery was obtained

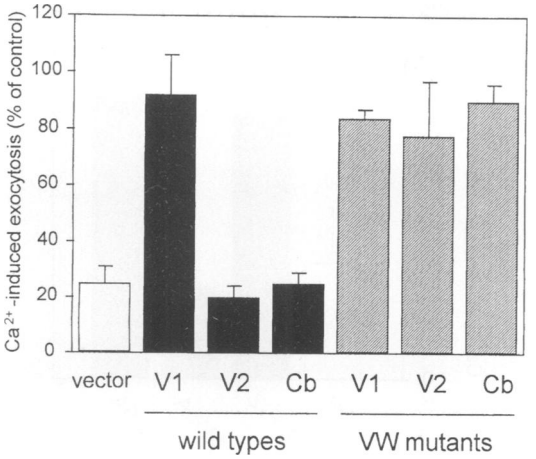

Fig. 2. Reconstitution of Ca^{2+}-induced exocytosis in streptolysin-O-permeabilized HIT-T15 cells treated with 50 nM TeTx. The cells of the hamster line HIT-T15 were transiently co-transfected with human proinsulin and with the wild-types or the VW mutants of VAMP-1 (V1), VAMP-2 (V2) or cellubrevin (Cb). Two days after transfection, the cells were permeabilized with SL-O for 7 min and incubated in the presence or absence of 50 nM purified light chain TeTx for a further 8 min. The medium was then removed and the cells stimulated by increasing the free Ca^{2+} concentration from 0.1 to 10 μM. Exocytosis from transfected cells was assessed by measuring the amount of human C-peptide released into the medium during the stimulation period. This figure indicates the amount of Ca^{2+}-induced secretion remaining after incubation of the cells with TeTx; 100% represents the Ca^{2+}-triggered secretion of cells transfected with the indicated constructs but incubated in the absence of TeTx. The results are the mean ± SD of at least three independent experiments.

in cells co-expressing both cellubrevinVW and VAMP-2VW (Figure 3).

Identification of the signal required for the targeting of VAMPs to insulin-containing secretory granules

The results obtained with the TeTx-resistant mutants of VAMP-2 and cellubrevin prompted us to analyze the domains of these proteins required to mediate exocytosis in insulin-secreting cells. An amino acid sequence within helix 1 is essential for the targeting of VAMPs to synaptic vesicles. Thus, a mutation of Met46 to Ala (M46A) prevents the targeting of VAMP-2 to synaptic vesicles (Grote *et al.*, 1995). Since the biogenesis of synaptic vesicles is different from that of dense core granules (Bauerfeind *et al.*, 1994), we determined whether the same signal was responsible for the targeting of VAMP-2 to synaptic vesicles and to insulin-containing secretory granules. For this purpose, we established a stable clone of INS-1 cells, a well granulated insulin-secreting line (Asfari *et al.*, 1992), expressing the epitope-tagged version of VAMP-2 (VAMP-2TAg) mutated at position 46 (M46A). When extracts of these cells were analyzed on sucrose density gradients, endogenous VAMP-2 co-sedimented both with synaptophysin (marker for synaptic-like vesicles) and with insulin (marker for dense core granules) (Figure 4). In contrast, the M46A mutant of VAMP-2 was recovered in one single population of vesicles with a density slightly higher than that of synaptic-like micro-vesicles and was undetectable in the fractions containing dense core granules (Figure 4).

The subcellular distribution of wild-type VAMP-2 and

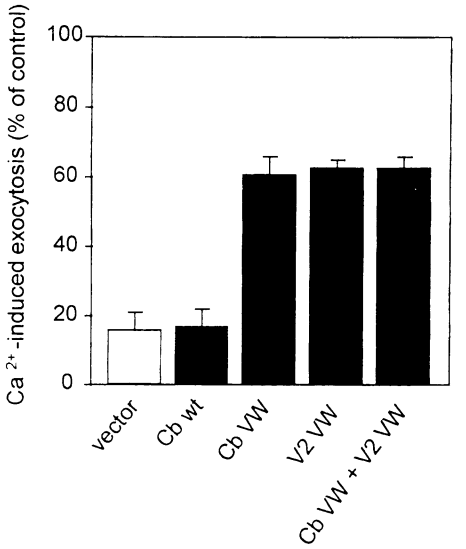

Fig. 3. Reconstitution of Ca^{2+}-induced exocytosis in HIT-T15 cells treated with 100 nM TeTx. HIT-T15 cells co-transfected with human proinsulin and with wild-type cellubrevin (Cb wt), the VW mutant of cellubrevin (Cb VW), the VW mutant of VAMP-2 (V2 VW) or both VAMP mutants were incubated in the presence of SL-O as described in Figure 2. The permeabilized cells were incubated in the presence of 100 nM light chain TeTx and stimulated as described in Figure 2. The figure shows the amount of human C-peptide released into the medium in response to 10 μM Ca^{2+} by the cells pre-incubated with TeTx; 100% represents the release of human C-peptide from cells transfected with each construct but incubated in the absence of the neurotoxin.

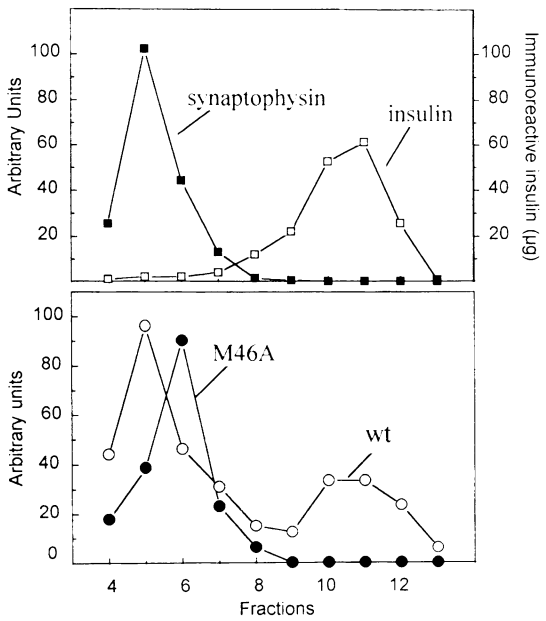

Fig. 4. Subcellular distribution of endogenous VAMP-2 and of the VAMP-2 M46A mutant. INS-1 cells were stably transfected with an epitope-tagged variant of VAMP-2 (VAMP-2TAg) in which Met46 was mutated to Ala (M46A). The cells were disrupted by nitrogen cavitation and the homogenate loaded on a continuous sucrose density gradient after separation of nuclei and cell debris. In the upper panel, aliquots of the fractions collected from the top of the gradient after 18 h centrifugation at 110 000 *g* were analyzed by Western blotting with an antibody against synaptophysin (■) or were processed for radioimmunoassay to quantify the amount of insulin (□) present. In the lower panel, the distribution of endogenous (wt, ○) and of mutated VAMP-2 (M46A, ●) was evaluated by Western blotting. Since the epitope-tagged M46A mutant of VAMP-2 is larger than the endogenous VAMP-2, both proteins could be analyzed on the same blot with an antibody against the N-terminus of VAMP-2.

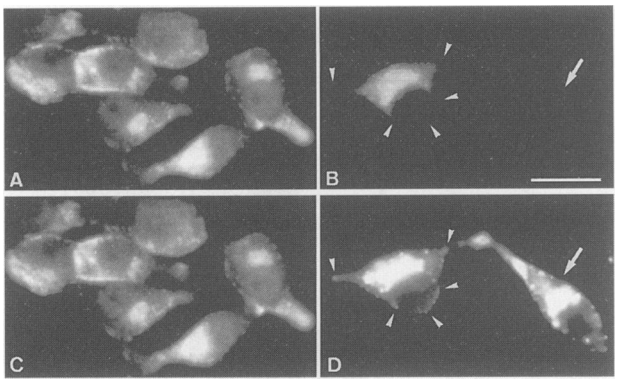

Fig. 5. Subcellular localization of wild-type VAMP-2 and of the M46A mutant by immunofluorescence light microscopy. Control INS-1 cells (**A** and **C**) and INS-1 cells transfected with the M46A mutant of VAMP-2TAg (**B** and **D**) were double stained with an antibody against VAMP-2 (A) or the T antigen epitope present in VAMP-2TAg (B) and with an antibody against insulin (C and D). Comparison of (A) and (C) shows that in non-transfected cells endogenous VAMP-2 was co-localized precisely with insulin. Comparison of (B) and (D) reveals that the transfected VAMP-2TAg M46A was located essentially in a perinuclear region and was not detectable in several insulin-containing granules (arrowheads). The arrow points to an insulin-containing cell which did not express detectable levels of the M46A mutant of VAMP-2. The bar represents 10 μM.

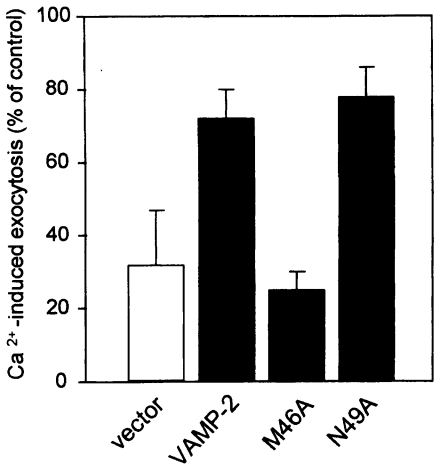

Fig. 6. The M46A mutant of VAMP-2 does not reconstitute Ca^{2+}-induced exocytosis in TeTx-treated HIT-T15 cells. HIT-T15 cells were transiently co-transfected with human proinsulin and with the TeTx-resistant (VW) form of VAMP-2TAg, the mutant M46A or the mutant N49A. After 2 days, the cells were permeabilized with SL-O and incubated in the presence or absence of TeTx as described in Figure 2. Ca^{2+}-induced exocytosis of transfected cells was assessed by measuring human C-peptide release. The results shown represent the amount of stimulated secretion obtained in the presence of TeTx and are expressed as a percentage of the C-peptide release measured in the absence of the neurotoxin. The results are the mean ± SD of at least three independent experiments.

of the M46A mutant was also analyzed by immunofluorescence. In INS-1 cells, endogenous VAMP-2 largely co-localizes with insulin (Figure 5A and C). An epitope-tagged mutant in which the transmembrane domain of VAMP-2 was replaced with the corresponding domain of the transferrin receptor displayed the same distribution (not shown). In contrast, the M46A mutant of VAMP-2 was found associated with vesicular structures concentrated in the perinuclear region but did not always co-localize with insulin (Figure 5B and D). This was particularly evident when secretory granules at the periphery of the cells were inspected (Figure 5). The level of expression of M46A varied and, in occasional cells, the protein was not detectable.

We then tested whether the TeTx-resistant variant of the M46A mutant of VAMP-2 (M46AVW) was able to rescue Ca^{2+}-mediated secretion in HIT-T15 cells. As shown in Figure 6, TeTx pre-treatment inhibited stimulated exocytosis in cells transfected with the vector alone by ~70%. In cells expressing the TeTx-resistant variant of VAMP-2, Ca^{2+}-evoked secretion was restored to 75–80% of the value obtained in cells incubated in the absence of the neurotoxin. By contrast, the TeTx-insensitive M46A mutant was unable to reconstitute secretion. Another VAMP-2VW mutant with a different substitution in helix 1, Asn49 to Ala (N49A), that displays an increased targeting to synaptic vesicles (Grote *et al.*, 1995), restored exocytosis as efficiently as VAMP-2VW (Figure 6).

Mapping of the domains of VAMP-2 essential to restore Ca^{2+}-triggered secretion

The domains of VAMPs essential for insulin exocytosis were identified using deletion mutants of VAMP-2TAg (Grote *et al.*, 1995). The mutants were first synthesized *in vitro* and incubated with TeTx as described in Figure 1. The mutants with a deletion between the amino acids 41 and 50 (Δ41–50) and between 71 and 80 (Δ71–80) were insensitive to TeTx treatment since they lack, respectively, the recognition motif for the toxin (Rossetto *et al.*, 1995) and the cleavage site (Schiavo *et al.*, 1992). To render all the other mutants resistant to proteolytic cleavage by the toxin, the amino acids corresponding to Gln76 and Phe77 were substituted by Val and Trp. After transient transfection in HIT-T15 cells, all the mutants were found to be expressed at comparable levels (not shown). The Δ2–31 mutant lacking the N-terminal variable domain of VAMP-2 was able to reconstitute exocytosis albeit slightly less efficiently than the full-length protein (Figure 7). By contrast, all the deletions within the domain conserved between the three VAMP isoforms rendered the VAMP-2 mutants unable to restore Ca^{2+}-stimulated exocytosis (Figure 7). Substitution of the transmembrane domain of VAMP-2 with that of the transferrin receptor did not affect the ability of the protein to mediate secretion in HIT-T15 cells (Figure 7).

Interaction between VAMP-2 mutants and t-SNAREs

Next we examined whether the reconstitution of exocytosis in TeTx-treated cells correlates with the ability of the VAMP-2 mutants to interact with the t-SNAREs SNAP-25 and syntaxin I (Figure 8). We found that the Δ41–50 and the Δ51–60 mutants of VAMP-2 were unable to bind efficiently to SNAP-25 and syntaxin I. The mutants with deletions in the N-terminal domain (Δ2–31 and Δ31–38) were capable of interacting with the t-SNAREs but their efficacy was reduced (Figure 8). Surprisingly, deletions in helix 2 (Δ61–70 and Δ71–80) did not prevent the association of the VAMP mutants with SNAP-25, and the amount of Δ71–80 recovered on SNAP-25-coated beads was even higher than that of wild-type VAMP-2 (Figure 8). However, the binding of Δ61–70 and Δ71–80 to syntaxin I beads was very poor, suggesting that the failure

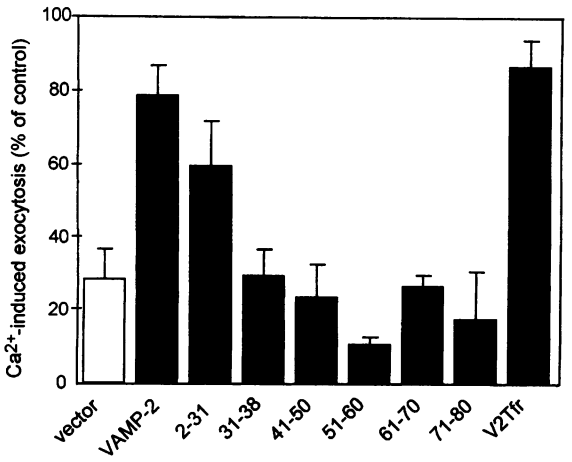

Fig. 7. Mapping of the domains of VAMP-2 required to restore Ca^{2+}-induced exocytosis in TeTx-treated HIT-T15 cells. HIT-T15 cells were transfected with human proinsulin and with the TeTx-resistant variants (VW) of VAMP-2TAg, Δ2–31, Δ31–38, Δ41–50, Δ51–60, Δ61–70, Δ71–80, and the chimera VAMP-2Tfr, in which the transmembrane domain of VAMP-2TAg is replaced by the transmembrane domain of the transferrin receptor (V2Tfr). The ability of each of these proteins to rescue Ca^{2+}-evoked secretion in TeTx-treated cells was determined as described in Figure 2. The results are the mean ± SD of at least three independent experiments.

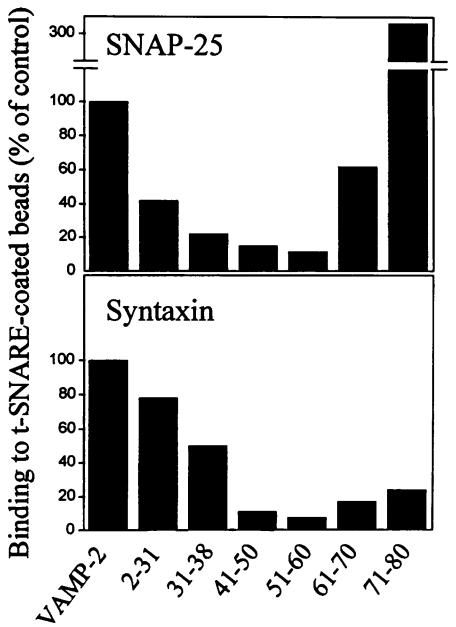

Fig. 8. Interaction between VAMP-2 mutants and t-SNAREs. Wild-type VAMP-2 and the indicated deletion mutants of the protein produced by *in vitro* translation were incubated in the presence of beads coated with either SNAP-25b (top panel) or syntaxin IA (bottom panel). The fraction of each VAMP-2 mutant attached to the beads was evaluated by phosphoimage analysis after SDS–PAGE and was compared with the wild-type protein (100%). The results are representative of two to four independent experiments.

of Δ61–70 and Δ71–80 to reconstitute Ca^{2+}-stimulated secretion is probably due to the lack of interaction between these VAMP-2 mutants and syntaxin.

Assessment of the involvement of VAMP-2 phosphorylation in the modulation of insulin secretion

Two potential phosphorylation sites present in VAMP-2, Thr35 and Ser61, are phophorylated *in vitro* by endogenous

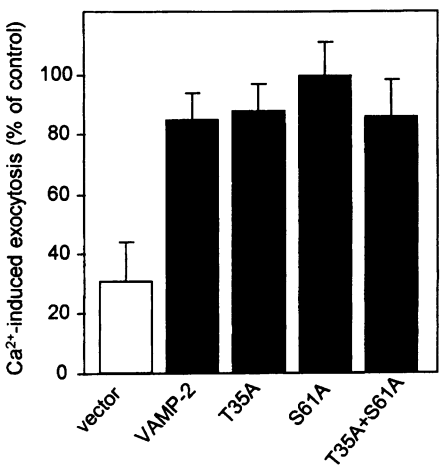

Fig. 9. Assessment of the role of VAMP-2 phosphorylation in the modulation of insulin secretion. HIT-T15 cells were co-transfected with human proinsulin and with the TeTx-resistant forms of VAMP-2TAg (VAMP-2), a mutant in which Thr35 was replaced with Ala (T35A), a mutant in which Ser61 was replaced with Ala (S61A) or a mutant in which both Thr35 and Ser61 were replaced with Ala (T35A+S61A). The ability of each of these proteins to rescue Ca^{2+}-evoked secretion in TeTx-treated cells was determined as described in Figure 2. The results are the mean ± SD of at least three independent experiments.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) associated with synaptic vesicles (Nielander *et al.*, 1995). We have tested the potential regulatory role of this reaction by replacing in VAMP-2VW one or both of the amino acids phosphorylated by CaMKII. The replacement of the potential phosphorylation sites with Ala did not significantly affect the ability of the protein to restore stimulated exocytosis (Figure 9).

Effect on VAMP-2 function of mutations in the SNARE motifs that prevent the binding of clostridial toxins

VAMPs, SNAP-25 and syntaxins contain a nine residue sequence, referred to as the SNARE motif, that functions as a recognition site for clostridial toxins (Rossetto *et al.*, 1994, 1995). Replacement of negative by positive charges in these motifs prevents the cleavage of VAMP-2 by TeTx and by BoNT B (Pellizzari *et al.*, 1996). To study whether clostridial toxins recognize a sequence motif that is normally required for the association of a regulatory factor, we tested the effect of the mutations that prevent the binding of TeTx or BoNT B on the ability of VAMP-2VW to restore exocytosis. Neither the D40N,E41Q nor the D64N,D65N mutations significantly affected the ability of VAMP-2VW to rescue Ca^{2+}-stimulated secretion (Figure 10). Similar results were obtained when Asp64 and Asp65 were replaced by two Ala residues (not shown).

Discussion

To analyze the role of VAMP-2 and cellubrevin in the control of insulin secretion, we have generated variants of the VAMP isoforms resistant to TeTx cleavage. Expression of these VAMP mutants restored Ca^{2+}-triggered exocytosis in insulin-secreting cells treated with TeTx, demonstrating that the proteolytic cleavage of VAMP-2 and cellubrevin is the main cause of the inhibition of

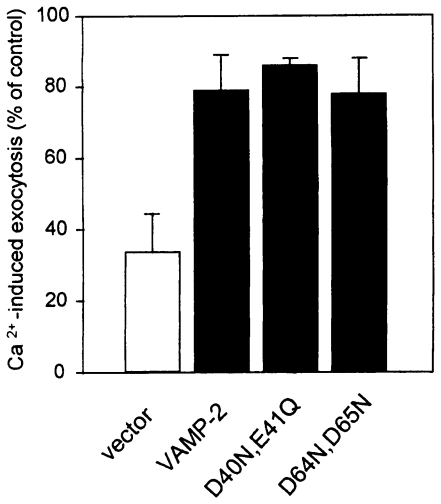

Fig. 10. Effect on VAMP-2 function of mutations in the SNARE motifs that prevent the binding of clostridial toxins. HIT-T15 cells were transiently co-transfected with human proinsulin and with either the TeTx-resistant forms of VAMP-2TAg (VAMP-2), a mutant in which Asp40 and Glu41 were replaced by Asn and Gln (D40N,E41Q), or a mutant in which both Asp64 and Asp65 were replaced by Asn (D64N,D65N). The ability of each of these proteins to rescue Ca^{2+}-evoked secretion in TeTx-treated cells was determined as described in Figure 2. The results are the mean ± SD of at least three independent experiments.

Ca^{2+}-evoked exocytosis observed in the presence of TeTx. Reconstitution of insulin exocytosis was almost complete in cells treated with 50 nM TeTx, but was less efficient in cells incubated in the presence of higher concentrations of the neurotoxin. The residual inhibition of secretion observed at high doses of TeTx could be due to the activation of transglutaminases (Ashton *et al.*, 1995), since stimulation of these enzymes has been reported to impair insulin release from pancreatic islets (Bungay *et al.*, 1984). Alternatively, because t-SNAREs contain copies of the amino acid motif recognized by clostridial neurotoxins (Rossetto *et al.*, 1994, 1995), high concentrations of TeTx could inhibit exocytosis by binding to SNAP-25 and/or to syntaxins thereby interfering with their function.

To rescue Ca^{2+}-stimulated exocytosis, the expression of VAMP-2 had to be adjusted to levels approaching those found in untransfected cells. High overexpression of VAMP-2 impaired stimulated exocytosis of HIT-T15 cells, an effect that may result from the same phenomenon previously observed after overexpression of syntaxin Ia and syntaxin 5 (Dascher and Balch, 1996; Nagamatsu *et al.*, 1996) and other unrelated proteins (Vozzi *et al.*, 1995). The mechanism underlying this dominant-negative effect is unknown, but may be explained by the depletion of the endogenous pool of a regulatory factor (Dascher and Balch, 1996).

Secretory granules of pancreatic β-cells contain VAMP-2 and cellubrevin (Regazzi *et al.*, 1995). Here, we show that the inhibition of Ca^{2+}-stimulated insulin secretion in TeTx-treated cells is restored with both VAMP isoforms. Ca^{2+}-evoked insulin secretion was also reconstituted in cells transfected with VAMP-1, which is normally not expressed in β-cells (Jacobsson *et al.*, 1994; Regazzi *et al.*, 1995; Rossetto *et al.*, 1996; Wheeler *et al.*, 1996), and with a VAMP-2 mutant (Δ2–31), in which the isoform-specific N-terminal domain was removed. This indicates that the different VAMP isoforms present on secretory vesicles can participate in the exocytotic process. The IgA protease of *Neisseria gonorrhoeae* cleaves VAMP-2 but not cellubrevin and blocks catecholamine release. For this reason, the secretory process of chromaffin cells has been proposed to require VAMP-2 but not cellubrevin (Binscheck *et al.*, 1995). However, since the IgA protease has a broader substrate specificity compared with clostridial neurotoxins, it cannot be excluded that the enzyme produced by *N.gonorrhoeae* cleaves, in addition to VAMP-2, other proteins implicated in the exocytotic process. In yeast, deletion of either SNC1 or SNC2, the two v-SNAREs associated with post-Golgi vesicles, does not affect secretion, but disruption of both of these genes prevents the fusion of the vesicles with the cell membrane (Protopopov *et al.*, 1993). A certain redundancy in the v-SNAREs is also suggested by studies performed with recombinant proteins that show that each of the three mammalian VAMP isoforms can form complexes with the t-SNAREs (Calakos *et al.*, 1994; Hayashi *et al.*, 1994; Chilcote *et al.*, 1995). It is conceivable that the presence of more than one VAMP isoform on secretory vesicles may be required for the fine tuning of the exocytotic process. Subtle differences in the affinity of v-SNAREs for t-SNAREs may influence the efficacy of the fusion of vesicles with the acceptor membrane and affect the secretory rate.

VAMPs are specifically associated with post-Golgi secretory vesicles (Chilcote *et al.*, 1995; Grote *et al.*, 1995; Regazzi *et al.*, 1995). The targeting of VAMP-2 and cellubrevin to synaptic vesicles requires a signal within a predicted amphipathic α-helix. Replacement of Met46 with Ala prevents the targeting of VAMP-2 to synaptic vesicles (Grote *et al.*, 1995) and, as shown here, to insulin-containing secretory granules. This suggests that although synaptic vesicles and dense core granules originate from different cellular compartments (Bauerfeind *et al.*, 1994), the same motif is required for VAMP targeting to both organelles. The precise mechanism permitting the recruitment of VAMPs to post-Golgi secretory vesicles remains to be investigated.

Much of what is known about the domains of VAMPs involved in the secretory process has been inferred from studies performed *in vitro* using recombinant proteins or by co-immunoprecipitation of v-SNAREs and t-SNAREs. However, *in vitro*, the interaction of VAMPs with SNAP-25 and syntaxins is analyzed in the absence of membranes (Calakos *et al.*, 1994; Hayashi *et al.*, 1994) and the majority of the complexes between v-SNAREs and t-SNAREs that are immunoprecipitated from detergent extracts are formed after cell lysis (Hayashi *et al.*, 1994). The assay for the reconstitution of stimulated insulin secretion that we have established overcomes these problems and permits a direct determination of the VAMP-2 domains required for the exocytotic process. Our results demonstrate that the domain of VAMP-2 from amino acids 51 to 80 is not essential for the targeting to secretory granules but is implicated in the interaction with the t-SNAREs and is required for insulin exocytosis. The Δ71–80 mutant of VAMP-2 that is inactive in the reconstitution assay binds SNAP-25 but not syntaxin I. This indicates that the interaction between VAMP-2 and SNAP-25 is not sufficient to sustain the secretory process and that, in agreement

with the model proposed from studies using recombinant proteins (Hayashi *et al.*, 1994), the formation of a ternary complex including VAMP-2, SNAP-25 and syntaxin I is required.

VAMPs contain potential phosphorylation sites in a domain essential for the interaction with t-SNAREs. Indeed, in purified synaptic vesicles, VAMP-2 is phosphorylated by CAMKII (Nielander *et al.*, 1995). We have investigated the implication of VAMP-2 phosphorylation in Ca^{2+}-mediated insulin secretion by mutating two potential phosphorylation sites to Ala. These mutations did not affect the ability of VAMP-2 to participate in exocytosis, suggesting that if VAMP-2 in insulin-secreting cells is indeed phosphorylated this reaction is not an essential requirement for the fusion process.

Substrate recognition by clostridial neurotoxins occurs through a nine amino acid motif that in mammalian cells is found exclusively in VAMPs, SNAP-25 and syntaxins (Rossetto *et al.*, 1994, 1995). The presence of this motif in three protein families all implicated in exocytosis raises the possibility that clostridial neurotoxins exploit for substrate recognition the same mechanism that is used by a component of the fusion machinery. However, substitutions in the negatively charged residues of the motifs that prevent substrate recognition by clostridial neurotoxins (Pellizzari *et al.*, 1996) did not impair the function of VAMP-2. Thus, these results indicate that the mechanism of substrate recognition by clostridial toxins does not mimic directly the interaction between VAMPs and other components of the secretory machinery.

In conclusion, we generated TeTx-resistant variants of VAMPs capable of restoring Ca^{2+}-stimulated exocytosis in insulin-secreting cells treated with the neurotoxin. This offered the opportunity in living cells of analyzing the effect on insulin exocytosis of amino acid deletions and substitutions in VAMP-2. A similar approach may be used in the future to define functionally important domains of SNAP-25 and of syntaxins. This reconstitution assay should complement the studies performed *in vitro* with recombinant proteins, thereby contributing to a better knowledge of the secretory process.

Materials and methods

Materials

The plasmids encoding the epitope-tagged rat VAMP-2 (VAMP-2TAg), the corresponding deletion mutants and the chimera VAMP-2 TfR-TM were prepared as described (Grote *et al.*, 1995). The cDNA coding for rat VAMP-1 was obtained from Dr C.Montecucco, University of Padua, Italy and rat cellubrevin from Dr T.Südhof, University of Texas, USA. The human proinsulin vector was kindly provided by Dr J.-C.Irminger, University of Geneva. Human CD4 cDNA was donated by Dr V.Steimle, University of Geneva. The antibody against VAMP-2 (Cl 69.1) was a kind gift from Dr R.Jahn, Yale University, USA. Purified TeTx light chain and recombinant SL-O were generously provided by Dr U.Weller, University of Mainz, Germany. TeTx was dialyzed against potassium glutamate buffer (see below).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the method of Kunkel *et al.* (1987), and the mutations were confirmed by DNA sequencing of the plasmids.

In vitro translation

In vitro transcription/translation was performed in reticulocyte lysates in the presence of [^{35}S]methionine using the protocol indicated by the manufacturer (Promega). An aliquot (5 µl) of the proteins produced *in vitro* was incubated in potassium glutamate buffer (20 mM HEPES, pH 7.0, 140 mM potassium glutamate, 5 mM NaCl, 7 mM $MgSO_4$, 5 mM Na_2ATP, 10.2 mM EGTA and Ca^{2+} to achieve a free Ca^{2+} concentration of 100 nM) at 37°C for 18 h. At the end of the incubation, the proteins were boiled for 5 min and separated by SDS–PAGE. The protein bands were visualized by autoradiography and quantified by densitometric scanning of the films.

Binding of VAMP deletion mutants to t-SNAREs

GST–SNAP-25b and GST–syntaxin IA fusion proteins were immobilized on glutathione–Sepharose beads as described (Chapman *et al.*, 1994). For each analysis, 25 µl of beads (dry bed volume) were incubated in 300 µl of binding buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA and 0.5% Triton X-100) containing 12 µl of the *in vitro* translated [^{35}S]methionine-labeled VAMP-2 mutant. After overnight incubation at 4°C, the beads were washed four times with 1 ml of binding buffer and boiled in 50 µl of sample buffer for SDS–PAGE containing β-mercaptoethanol. The proteins attached to the beads and an aliquot of the *in vitro* translation product were subjected to SDS–PAGE. For the evaluation of the fraction of labeled VAMP proteins bound to the beads, the gel was exposed to a PhosphoImager screen (Molecular Dynamics).

Cell culture and transfection

The insulin-secreting cell lines HIT-T15 and INS-1 were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and other additions as described (Regazzi *et al.*, 1990; Asfari *et al.*, 1992). For transient transfection experiments, HIT-T15 cells were seeded in 24 multiwell plates (3.5×10^5 cells/well). After 4 days of culture, the cells were co-transfected using the lipopolyamine Transfectam (Promega) with 3 µg of the vector encoding human proinsulin and 1.5 µg of the plasmids containing the cDNAs under study (Lang *et al.*, 1995; Regazzi *et al.*, 1996).

Purification of transfected cells

HIT-T15 cells (2×10^6) seeded in 21 cm^2 Petri dishes were transiently transfected with 7 µg of the constructs under study, 13 µg of pcDNA3 encoding human CD4 and 4 µl of Transfectam. Two days after transfection, the cells were detached by trypsinization and incubated for 1 h at 4°C in culture medium in the presence of magnetic beads ($\sim1.4\times10^6$ beads) coated with an anti-CD4 antibody (Dynal). The transfected cells expressing human CD4 attached to the beads were washed three times with phosphate-buffered saline (PBS; 137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.5 mM $MgCl_2$ and 0.7 mM $CaCl_2$, pH 7.5) supplemented with 2% FCS and resuspended in TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA and 5 µg/ml aprotinin). The cells were disrupted by sonication and a crude membrane fraction prepared by centrifuging the extract for 30 min at 14 000 *g*. The pellet was resuspended in TE buffer supplemented with 0.5% Triton-X-100 and recentrifuged for 30 min at 14 000 *g*. The supernatant was collected and analyzed by SDS–PAGE. The ratio between the amount of endogenous and transfected VAMPs was estimated by Western blotting followed by densitometric scanning of the films.

Cell permeabilization and secretion assays

Transfected HIT-T15 cells were washed twice with Krebs–Ringer buffer and permeabilized for 7 min at 37°C with SL-O in potassium glutamate buffer (Regazzi *et al.*, 1995). The permeabilized cells were incubated for 8 min at 37°C in the presence or absence of 50 nM TeTx light chain. The medium was then removed and exocytosis triggered by increasing the free Ca^{2+} concentration from 0.1 to 10 µM. Secretion from transfected cells (10–15%) was assessed by measuring, by enzyme-linked immunosorbent assay (ELISA; Dako), the amount of human C-peptide released into the medium. This ELISA assay does not cross-react with endogenous hamster C-peptide produced by HIT-T15 cells. Secretion from the whole population of cells present in the well was determined by assaying the total amount of insulin (hamster plus human) released into the medium by radioimmunoassay.

Subcellular fractionation

Two 155 cm^2 Petri dishes of INS-1 cells stably transfected with an epitope-tagged variant of VAMP-2 mutated at position 46 (Met to Ala) (Grote *et al.*, 1995) were homogenized by nitrogen cavitation (Regazzi *et al.*, 1995). Synaptic-like microvesicles and insulin-containing granules were separated by centrifugation (18 h at 110 000 *g*) on a continuous sucrose density gradient (0.45–2 M) (Reetz *et al.*, 1991; Regazzi *et al.*, 1995).

Immunofluorescence

INS-1 cells cultured on glass coverslips coated with bovine endothelial matrix (Eldan Tech., Jerusalem, Israel) were rinsed in PBS and were fixed in 4% paraformaldehyde for 20 min. After one wash with PBS and two washes in PBS containing 0.38% glycine and 0.27% NH_4Cl, the cells were permeabilized in PBS supplemented with 0.5% bovine serum albumin and 0.1% saponin for 30 min (blocking solution). The cells were incubated with the antibodies for 1 h at room temperature. After five washes with blocking solution, the cells were incubated for 45 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and/or with rhodamine-conjugated goat anti-hamster antibodies. The coverslips were rinsed five times in blocking solution and mounted for analysis.

Acknowledgements

We should like to thank Dr C.Montecucco, University of Padua, for critical discussions and helpful suggestions. We are grateful to Dr U.Weller, University of Mainz for the generous supply of streptolysin-O and of purified tetanus toxin light chain. We also thank Dr R.Jahn, Yale University, for the antibody against VAMP-2, Dr T.Südhof, University of Texas, for the cellubrevin cDNA, Dr R.Scheller, Stanford University, for the plasmid encoding recombinant syntaxin Ia, Dr J.-C.Irminger, University of Geneva, for the plasmid encoding human proinsulin and Dr V.Steimle, University of Geneva, for the human CD4 cDNA. We gratefully acknowledge the expert technical assistance of Ms Dominique Duhamel and Mr Stéphane Dupuis. This work was supported by the Juvenile Diabetes Foundation International Research Grants (R.R., P.M. and P.A.H.), by the Swiss National Science Foundation Grants No. MHV-32-45000.95 (K.S.), 32-43086.95 (P.M.), 31-40839.94 (P.A.H.) and 32-32376.91 (C.B.W.), by Glaxo Research and Development Ltd (K.S. and P.A.H.) and by the European Union (P.M.).

References

Asfari,M., Janjic,D., Meda,P., Li,G., Halban,P.A. and Wollheim,C.B. (1992) Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology*, **130**, 167–178.

Ashton,A.S., Li,Y., Doussau,F., Weller,U., Dougan,G., Poulain,B. and Dolly,J.O. (1995) Tetanus toxin inhibits neuroexocytosis even when its Zn^{2+}-dependent protease activity is removed. *J. Biol. Chem.*, **270**, 31386–31390.

Bauerfeind,R., Ohashi,M. and Huttner,W.B. (1994) Biogenesis of secretory granules and synaptic vesicles. Facts and hypothesis. *Ann. N.Y. Acad. Sci.*, **733**, 233–244.

Binscheck,T., Bartels,F., Bergel,H., Bigalke,H., Yamasaki,S., Hayashi,T., Niemann,H. and Pohlner,J. (1995) IgA protease from *Neisseria gonorrhoeae* inhibits exocytosis in bovine chromaffin cells like tetanus toxin. *J. Biol. Chem.*, **270**, 1770–1774.

Bungay,P.J., Potter,J.M. and Griffin,M. (1984) The inhibition of glucose-stimulated insulin secretion by primary amines. A role for transglutaminase in the secretory process. *Biochem. J.*, **219**, 819–827.

Calakos,N., Bennett,M.K., Peterson,K.E. and Scheller,R.H. (1994) Protein–protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science*, **263**, 1146–1149.

Chapman,E.R., An,S., Barton,N. and Jahn,R. (1994) SNAP-25, a t-SNARE which binds to both syntaxin and synaptobrevin via domains that may form coiled coils. *J. Biol. Chem.*, **269**, 27427–27432.

Chilcote,T.J., Galli,T., Mundigl,O., Edelmann,L., McPherson,P.S., Takei,K. and De Camilli,P. (1995) Cellubrevin and synaptobrevins: similar subcellular localization and biochemical properties in PC12. *J. Cell Biol.*, **129**, 219–231.

Dascher,C. and Balch,W.E. (1996) Mammalian Sly1 regulates syntaxin 5 function in endoplasmic reticulum to Golgi transport. *J. Biol. Chem.*, **271**, 15866–15869.

Dascher,C., Ossig,R., Gallwitz,D. and Schmitt,H.D. (1991) Identification and structure of four yeast genes (SLY) that are able to suppress the functional loss of YPT1, a member of the RAS superfamily. *Mol. Cell. Biol.*, **11**, 872–885.

Elferink,L.A., Trimble,W.S. and Scheller,R.H. (1989) Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system. *J. Biol. Chem.*, **264**, 11061–11064.

Ferro-Novick,S. and Jahn,R. (1994) Vesicle fusion from yeast to man. *Nature*, **370**, 191–193.

Galli,T., Chilcote,T., Binz,T., Niemann,H. and De Camilli,P. (1994) Tetanus toxin-mediated cleavage of cellubrevin impairs exocytosis of transferrin receptor-containing vesicles in CHO cells. *J. Cell Biol.*, **125**, 1015–1024.

Grote,E., Hao,J.C., Bennett,M.K. and Kelly,R.B. (1995) A targeting signal in VAMP regulating transport to synaptic vesicles. *Cell*, **81**, 581–589.

Hayashi,T., McMahon,H., Yamasaki,S., Binz,T., Hata,Y., Sudhof,T.C. and Niemann,H. (1994) Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *EMBO J.*, **13**, 5051–5061.

Jacobsson,G., Bean,A.J., Scheller,R.H., Juntti-Berggren,L., Deeney,J.T., Berggren,P.-O. and Meister,B. (1994) Identification of synaptic proteins and their isoform mRNAs in compartments of pancreatic endocrine cells. *Proc. Natl Acad. Sci. USA*, **91**, 12487–12491.

Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–382.

Lang,J., Nishimoto,I., Okamoto,T., Regazzi,R., Kiraly,C., Weller,U. and Wollheim,C.B. (1995) Direct control of exocytosis by receptor-mediated activation of the heterotrimeric GTPases Gi and Go or by the expression of their active Ga subunits. *EMBO J.*, **14**, 3635–3644.

Lupas,A., Van Dyke,M. and Stock,J. (1991) Predicting coiled coils from protein sequences. *Science*, **252**, 1162–1164.

McMahon,H.T., Ushkaryov,Y.A., Edelman,L., Link,E., Binz,T., Niemann,H., Jahn,R. and Südhof,T.C. (1993) Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature*, **364**, 346–349.

Nagamatsu,S., Fujiwara,T., Nakamichi,Y., Watanabe,T., Katahira,H., Sawa,H. and Akagawa,K. (1996) Expression and functional role of syntaxin 1/HPC-1 in pancreatic beta cells: syntaxin 1A, but not 1B, plays a negative role in regulatory insulin release pathway. *J. Biol. Chem.*, **271**, 1160–1165.

Nielander,H.B., Onofri,F., Valtorta,F., Schiavo,G., Montecucco,C., Greengard,P. and Benfenati,F. (1995) Phosphorylation of VAMP/synaptobrevin in synaptic vesicles by endogenous protein kinases. *J. Neurochem.*, **65**, 1712–1720.

Niemann,H., Blasi,J. and Jahn,R. (1994) Clostridial neurotoxins: new tools for dissecting exocytosis. *Trends Cell Biol.*, **4**, 179–185.

Pellizzari,R., Rossetto,O., Lozzi,L., Giovedì,S., Johnson,E., Shone,C.C. and Montecucco,C. (1996) Structural determinants of the specificity for VAMP/synaptobrevin of tetanus and botulinum type B and G neurotoxins. *J. Biol. Chem.*, **271**, 20353–20358.

Protopopov,V., Govindan,B., Novick,P. and Gerst,J.E. (1993) Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S.cerevisiae*. *Cell*, **74**, 855–861.

Reetz,A., Solimena,M., Matteoli,M., Folli,F., Takei,K. and De Camilli,P. (1991) GABA and pancreatic β-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *EMBO J.*, **10**, 1275–1284.

Regazzi,R., Li,G., Deshusses,J. and Wollheim,C.B. (1990) Stimulus–response coupling in insulin-secreting HIT cells. *J. Biol. Chem.*, **265**, 15003–15009.

Regazzi,R. *et al.* (1995) VAMP-2 and cellubrevin are expressed in pancreatic β-cells and are essential for Ca^{2+}- but not for GTPγS-induced insulin secretion. *EMBO J.*, **14**, 2723–2730.

Regazzi,R., Ravazzola,M., Iezzi,M., Lang,J., Zahraoui,A., Andereggen,E., Morel,P., Takai,Y. and Wollheim,C.B. (1996) Expression, localization and functional role of small GTPases of the Rab3 family in insulin-secreting cells. *J. Cell Sci.*, **109**, 2265–2273.

Rossetto,O., Schiavo,G., Montecucco,C., Poulain,B., Deloye,F., Lozzi,L. and Shone,C.C. (1994) SNARE motif and neurotoxins. *Nature*, **372**, 415–416.

Rossetto,O., Deloye,F., Poulain,B., Pellizzari,R., Schiavo,G. and Montecucco,C. (1995) The metallo-proteinase activity of tetanus and botulism neurotoxins. *J. Physiol.*, **89**, 43–50.

Rossetto,O., Gorza,L., Schiavo,G., Schiavo,N., Scheller,R.H. and Montecucco,C. (1996) Vamp/synaptobrevin isoforms 1 and 2 are widely and differentially expressed in nonneuronal tissues. *J. Cell Biol.*, **132**, 167–179.

Rothman,J.E. (1994) Mechanisms of intracellular protein transport. *Nature*, **372**, 55–63.

Rothman,J.E. and Warren,G. (1994) Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr. Biol.*, **4**, 220–233.

Sadoul,K., Lang,J., Montecucco,C., Weller,U., Regazzi,R., Catsicas,S., Wollheim,C.B. and Halban,P.A. (1995) SNAP-25 is expressed in islets

of Langerhans and is involved in insulin release. *J. Cell Biol.*, **128**, 1019–1028.

Schiavo,G., Benfenati,F., Poulain,B., Rossetto,O., Polverino de Laureto,P., DasGupta,B.R. and Montecucco,C. (1992) Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature*, **359**, 832–835.

Söllner,T., Whiteheart,S.W., Brunner,M., Erdjument-Bromage,H., Geromanos,S., Tempst,P. and Rothman,J.E. (1993a) SNAP receptors implicated in vesicle targeting and fusion. *Nature*, **362**, 318–324.

Söllner,T., Bennett,M.K., Whiteheart,S.W., Scheller,R.H. and Rothman, J.E. (1993b) A protein assembly–disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell*, **75**, 409–418.

Vozzi,C., Ullrich,S., Charollais,A., Philippe,J., Orci,O. and Meda,P. (1995) Adequate connexin-mediated coupling is required for proper insulin production. *J. Cell Biol.*, **131**, 1561–1572.

Wheeler,M.B. *et al.* (1996) Characterization of SNARE protein expression in β cell lines and pancreatic islets. *Endocrinology*, **137**, 1340–1348.

Wollheim,C.B., Lang,J. and Regazzi,R. (1996) The exocytotic process of insulin secretion and its regulation by Ca^{2+} and G-proteins. *Diabetes Rev.*, **4**, 276–297.

Received on August 14, 1996; revised on September 11, 1996